Application of new bioformulations of *Pseudomonas aureofaciens* for biocontrol of cotton seedling damping-off

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Abstract: *Pseudomonas aureofaciens* (30-84) is a phenazine producing bacterium and reported as asuccessful biocontrol agent of some plant fungal pathogens. In the present study, the possibility of biological control of cotton damping-off caused by *Rhizoctonia solani* (AG-4) through phenazine production by the 30-84 strain, was investigated. In the search for the development of bioformulations of Pa (m) ($PhzR^-$) and Pa (w) ($PhzR^+$) strains of 30-84, four new carriers including soybean meal (SM), cottonseed meal (CM), rice bran (RB), and talc powder (TAL) were selected. The efficacy of bacterial formulations in reducing disease incidence was evaluated in four intervals (15, 30, 45, and 60 days after sowing), and compared with each bacterial suspension efficacy under green-house conditions. The results revealed that organic carriers were more effective than talc powder. It was also found that all the bioformulations were more efficient than each bacterial suspension. The most effective in reducing disease incidence was Pa (w) + RB. In contrast, Pa (m), Pa (m) + TAL, and Pa (m) + RB did not significantly suppress the disease in comparison with the infested control. Thus, phenazine production as a main biocontrol mechanism of *P. aureofaciens* (30-84) may be affected by the kind of carriers used for the bioformulation development.

Key words: bioformulation, cottonseed meal, phenazine, seedling mortality, soybean meal

Introduction

Cotton is an important cash crop cultivated in many countries around the world including Iran (Zaki *et al*. 1998; Heydari *et al*. 2007). Cotton seedlings are usually attacked by several soil-borne pathogenic fungi including *Rhizoctonia solani*. Among anastomosis groups of *R. solani* causing cotton damping-off, AG-4 is the most serious and exists world-wide, including Iran (Zaki *et al*. 1998; Naraghi *et al*. 2007). Although the most common method for controlling this disease is seed treatment with chemical fungicides, the potential harmful effects of such chemicals on human health, non-target organisms, and the environment have become a subject of public concern and a concern of environmental protection agencies. The above-mentioned concerns and problems have resulted in searching for an alternative, environmentally safe, and non-chemical strategy such as biological control (Cook 2000; Heydari and Ghredaghli 2007).

In recent years, biological control of plant diseases have included the use of beneficial fungi and bacteria. It is particularly bacteria which has become the subject of numerous research projects among agricultural scientists (Pierson III *et al*. 1994; Raupach and Kloepper 1998; Weller 1998; Samavat *et al*. 2008; Samavat *et al*. 2011; Heydari and Naraghi 2012). Several species of antagonistic bacteria including *Bacillus* and *Pseudomonas* have recently been used against different pathogenic fungi and have produced promising results (Shahraki *et al*. 2009; Heydari and Pessarakli 2010). The most common species of *Pseudomonas* bacteria which have been used in the biocontrol of plant diseases include *P. fluorescens*, *P. putida*, and *P. aureofaciens* (Wood *et al*. 1997; Weller 2007). These beneficial rhizobacteria can suppress the growth of multiple soil-borne fungi on different crops by using different antagonistic mechanisms such as antibiosis (Thomashow and Weller 1990; Shahraki *et al*. 2009; Heydari and Pessarakli 2010; Jamalizadeh *et al*. 2011).

P. aureofaciens strain 30-84 is a phenazine producing bacterium which can effectively suppress wheat take-all disease (Pierson and Thomashow 1992; Pierson III *et al*. 1994). The ability of 30-84 to biocontrol *Gaeumannomyces graminis* var. *tritici* correlates directly with its capacity to produce phenazine antibiotics (Pierson III and Thomashow 1992). Additionally, phenazine antibiotics play important roles in the competitive fitness and survival of this strain in the wheat rhizosphere (Mazzola *et al*. 1992). Phenazines may contribute directly in the successful control of other soil-borne plant diseases by the 30-84 strain

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(Pierson III *et al*. 1994; Chin-A-Woeng *et al*. 2003; Madulla *et al*. 2008). A functional gene of 30-84 named *phzR* (phenazine regulator) can regulate the expression of phenazine biosynthetic genes in response to environmental and bacterial population density signals (Pierson III *et al*. 1994). Many researchers use the *phzR* mutant (*phzR*$^-$) to study the phenazine role in biocontrol bacterial efficiency.

Biocontrol active microorganisms including antagonistic bacteria, usually perform effectively in the greenhouse and in controlled environments, but fail to do so in the field (Amer and Utkhede 2000; Cook 2000). One factor for the failure could be the lack of the proper formulations.

To introduce an effective and environmentally safe strategy for controlling cotton seedling mortality disease using novel biocontrol active agents, we tried to develop some bioformulations of *P. aureofaciens* with some new carriers. In this way, the efficiency of developed bioformulations was compared with each bacterial suspension. Furthermore, the possibility of the biocontrol of *R. solani* (AG-4), the casual agent of cotton seedling damping-off, through phenazine production by *P. aureofaciens* (30-84), was investigated.

Materials and Methods

Source of microorganisms and culture conditions

In this study, both *P. aureofaciens* strains, PhzR$^+$ [30-84 wild type, Pa (w)], and PhzR$^-$ [phenazine regulator mutant, Pa (m)], were obtained from the Department of Plant Pathology and Microbiology, College of Bioenvironmental Science, the University of Arizona, USA. Bacterial strains were stored in a 0.1 M magnesium sulfate ($MgSO_4$ × × $7H_2O$) solution at room temperature (22°C). The strains were cultivated in nutrient broth (NB) (Merck, Germany) and stored in broth containing 15% glycerol at −20°C for short-term preservation.

Three isolates (Co-1, Co-2, Co-3) of *R. solani* (AG-4) which were used in this study, were isolated from the root and crown of cotton. These fungal isolates were obtained from Microbial Culture Collection, Microorganisms Research Laboratory, Iranian Research Institute of Plant Protection, Tehran, Iran, with proven pathogenicity on cotton. The fungi were routinely grown on standard Potato Dextrose Agar (PDA) (Merck, Germany) and stored in broth containing 15% glycerol at −20°C.

Dual culture inhibition test

To test the efficiency of both wild type and mutant strains in inhibiting the growth of *R. solani* (AG-4) isolates under *in vitro* conditions, the dual culture test was carried out according to Hagedorn *et al*. 1989. Briefly, each bacterial strain was streaked along a line crossing the center of a Petri dish containing PDA medium. After 48 h, two 5-mm-mycelial discs of 2-day-old *R. solani* isolate culture in PDA were placed at both opposite sides of the Petri dish. The mycelial growth of the fungus toward the bacterial streak was measured whenever the fungal colony in the control completely encircled the Petri dish. This study included three replicates for each treatment and was repeated in two independent trials.

Percentage inhibition was calculated using the following formula (Sivan *et al.* 1987):

$$\% \text{ Inhibition} = [1 - (\text{fungal growth/control growth})] \times 100.$$

Preparation of bacterial suspension

From the 48 h culture of each bacterial strain in Nutrient Agar (NA) medium, one loop was inoculated into King's B broth medium and incubated on a shaker incubator at 150 rpm for 48 h at room temperature (28±2°C). Afterwards, bacterial cell density was adjusted to 10^9 CFU/ml at 620 nm and the obtained suspension was used for the preparation of the bioformulations.

Preparation of carriers for bioformulations

Respectively, talc powder and rice bran were selected as common inorganic and organic carriers. Additionally, the potentiality of two new organic compounds (soybean meal and cottonseed meal) was assessed for the preparation of *P. aureofaciens* 30-84 wild type and PhzR mutant bioformulations. All the above-mentioned carriers were steam-sterilised at 140 kPa for 30 min, and dried aseptically in glass trays for 12 h at 50°C before use (Jorjani *et al*. 2012).

Development of bioformulations

For the development of four replicates of each bioformulation, 50 g of each carrier was mixed with 50 ml of each bacterial suspension containing 10^9 CFU/ml. Then, 1 g carboxymethyl cellulose (CMC adhesive) was added to the mixture under sterile conditions (Vidhyasekaran and Muthuamilan 1995). The product was shade-dried to reduce the moisture content (less than 20%), packed in polypropylene bags and sealed. In each gram of bioformulations, the number of bacteria was 5×10^8 CFU. The ingredients of each bioformulation are shown in table 1.

Seed coating with the developed bioformulations

For this purpose, Varamin cultivar of cotton was obtained from the Cotton Research Institute, Varamin, Iran. For four replicates of a treatment, 48 seeds were initially surface-sterilised with 1% sodium hypochlorite and washed twice with distilled water. Afterwards, the cotton seeds were dried and added to Petri dishes containing one of the prepared bioformulations. The seeds were rolled in the mixture for about 10 min until they were completely coated with the bioformulation. The average number of bacteria on the surface of each treated cotton seed was about 10^8 CFU.

Evaluation of the antagonistic effects of bioformulations as seed treatment on cotton damping-off disease in the greenhouse

A pot culture study was carried out with the following procedures: soil was collected from a cotton field around a Tehran province. The soil was passed through a 3-mm

Table 1. Description of bioformulations developed in this study

Treatments	Bioformulation ingredients
Pa (w) + TAL	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^+$) (50 ml) containing 10^9 CFU/ml + talc powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (w) + RB	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^+$) (50 ml) containing 10^9 CFU/ml + rice bran powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (w) + SM	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^+$) (50 ml) containing 10^9 CFU/ml + soybean meal powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (w) + CM	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^+$) (50 ml) containing 10^9 CFU/ml + cottonseed meal powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (m) + TAL	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^-$) (50 ml) containing 10^9 CFU/ml + talc powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (m) + RB	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^-$) (50 ml) containing 10^9 CFU/ml + rice bran powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (m) + SM	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^-$) (50 ml) containing 10^9 CFU/ml + soybean meal powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds
Pa (m) + CM	Suspension of *P. aureofaciens* strain 30-84 (PhzR$^-$) (50 ml) containing 10^9 CFU/ml + cottonseed meal powder (50 g) + + 10% CMC (5 ml) + 48 cotton seeds

TAL – talc powder; RB – rice bran; SM – soybean meal; CM – cottonseed meal; CMC – carboxymethyl cellulose

sieve, air-dried and pasteurised with a steam heater for 6 h at 85°C. Each pot (20 cm in diameter) was filled with soil (3.5 kg) and infested with six one-cm discs of *R. solani* Co-1 as fungal inoculums according to Heydari and Misagh (1998). As above mentioned, 12 cotton seeds were treated with the various bioformulations, bacterial suspensions or Carboxin-Thiram (5 g/1,000 g seed) and sown (depth 2 cm; spacing 2 × 3 cm) in each pot. An infested control (infested soil + untreated seeds) as a control (+), and a non-infested control (pasteurised soil + untreated seeds) as a control (-) were also included. The pots were irrigated from above, every other day. For measuring the disease incidence, the number of emerged seedlings and the percentage of disease incidence were recorded during 15, 30, 45, and 60 days after sowing the cotton seeds. This study included four replicates for each treatment and was repeated in two independent trials. The percentage of disease incidence was calculated using the following formula:

$$\% \text{ Disease incidence} = [1 - (\text{the number of treatment-emerged seedlings/the number of the control (-) emerged seedlings})] \times 100.$$

Statistical analyses

All the experiments were performed in a completely randomised design. Analyses of variance (ANOVA) were carried out using the MSTAT-C, 1991 program version 2.10. Comparisons of the means were conducted using the Least Significant Differences (LSD) ($p = 0.05$).

Results

Dual culture test

The antibiosis activity of *P. aureofaciens*, Pa (w) (PhzR$^+$) and Pa (m) (PhzR$^-$), was tested against *R. solani* isolates and the results are indicated in figure 1. As shown in figure 1, the highest percentage of growth inhibition (67.56%) was related to *R. solani* (Co-1) affected by Pa (w). In all cases, Pa (w) inhibited the colony growth of the fungus more than Pa (m). It was found that phenazine antibiotics had an important effect on the inhibition of *R. solani* mycelial growth.

Greenhouse study

For the greenhouse study, *R. solani* (Co-1) which was the most pathogenic among the three isolates, was selected. The results of the effects of bioformulations on cotton seedling damping-off during the time period of 15, 30, 45, and 60 days after sowing are shown in table 2. According to this table, bioformulations Pa (w) + RB and Pa (w) + CM were the most effective with 6.25 and 12.50% disease incidence, respectively, 15 days after sowing. However, from among eight bioformulations, only two of them [Pa (m) + + TAL and Pa (m) + RB], did not show significant effects on the reduction of disease incidence in comparison with the inoculated control.

In the 30, 45, and 60 days after sowing intervals, the results were different in comparison with the first interval. In these intervals, all bioformulations performed effectively in controlling the damping-off disease, and showed a significant reduction in the disease incidence compared with the inoculated control. The most effective bioformulations included Pa (w) + RB and Pa (w) + CM with only 6.25 and 14.58% of disease, respectively.

Based on the results of greenhouse experiment which were presented in table 2, in all sampling intervals, most bioformulations developed by the wild type strain of *P. aureofaciens* showed more effectiveness in controlling cotton seedling damping-off disease in comparison with the commonly used fungicide Carboxin-Thiram. However, in the case of mutant strain, no bioformulation containing this strain showed significant difference in disease control compared with Carboxin-Thiram.

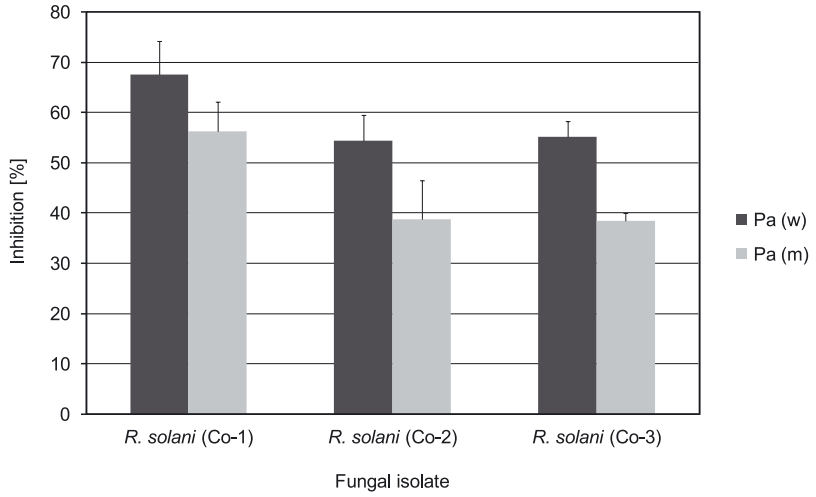

Fig. 1. Antibiosis activity of bacterial strains [*P. aureofaciens* Pa (w) and *P. aureofaciens* Pa (m)] against *R. solani* isolates (Co-1, Co-2, and Co-3)

Table 2. The efficacy of developed bioformulations on the percentage of cotton seedling mortality incidence and the average number of emerged seedlings 15, 30, 45, and 60 days after sowing in the greenhouse

Treatment	Disease incidence [%] 15th day	Disease incidence [%] 30th day	Disease incidence [%] 45th day	Disease incidence [%] 60th day
Control (-)	0 k	0 g	0 g	0 g
Control (+)	58.33±0.41 a	70.83±0.36 a	70.83±0.36 a	70.83±0.36 a
Fungicide	33.33±0.64 defg	35.42±0.28 cd	35.42±0.28 cd	35.42±0.28 cd
Pa (w)	33.33±0.20 defg	35.42±0.22 cd	35.42±0.22 cd	35.42±0.22 cd
Pa (w) + TAL	25.00±0.18 fghi	25.00±0.43 ed	25.00±0.43 ed	25.00±0.43 ed
Pa (w) + RB	6.25±0.17 jk	6.25±0.56 fg	6.25±0.56 fg	6.25±0.56 fg
Pa (w) + SM	18.75±0.24 ghij	18.75±0.16 def	18.75±0.16 def	18.75±0.16 def
Pa (w) + CM	12.50±0.37 hijk	14.58±0.34 efg	14.58±0.34 efg	14.58±0.34 efg
Pa (m)	58.33±0.28 a	70.83±0.26 a	70.83±0.26 a	70.83±0.26 a
Pa (m) + TAL	47.92±0.18 abc	52.08±0.15 b	52.08±0.15 b	52.08±0.15 b
Pa (m) + RB	52.08±0.21 ab	52.08±0.27 b	56.25±0.22 b	56.25±0.22 b
Pa (m) + SM	35.42±0.23 cdef	41.67±0.39 bc	41.67±0.39 bc	41.67±0.39 bc
Pa (m) + CM	39.58±0.36 bcde	41.67±0.46 bc	43.75±0.35 bc	43.75±0.35 bc

Each value is the average of four replicates in two independent greenhouse trials. In each column, values marked with the same letters are not statistically different according to Duncan's multiple range test ($p = 0.05$)

Discussion

The overall results of this study indicate that *P. aureofaciens*, particularly the wild type strain, and the bioformulations containing this bacterium, and organic carriers could effectively control cotton seedling damping-off causal agent in the laboratory and in greenhouse conditions.

The dual-culture assay with *R. solani* isolates and both wild type and phenazine mutant of *P. aureofaciens* strain 30-84, supported the fact that phenazine antibiotics play an important role in suppressing the growth of fungal colony. These findings are in agreement with the results obtained by Pierson and Thomashow (1992). They reported that *G. graminis* var. *tritici*, the causal agent of wheat take-all, was suppressed through the production of phenazine antibiotics by *P. aureofaciens* strain 30-84. Although the main Pa (w) mechanism for biocontrol of cotton damping-off caused by *R. solani* (AG-4) detected as phenazine production, the partial inhibition of fungal growth by Pa (m) was probably because of the production of other antagonistic metabolites including other antibiotics, which also applies to the results of our study. Furthermore, it was found that as the pathogenicity of the fungal isolate increased, the inhibition ability of its growth by antagonistic bacteria was also increased.

In the greenhouse experiment of the present study, the effectiveness of the bioformulations on the biocontrol of cotton seedling damping-off disease was evaluated in four different intervals. We chose these four time intervals, because, we wanted to observe the disease progress and the trend of the effects of the bioformulations. As the results show, there were no significant differences in the disease incidence and the performance of the bioformulations during different intervals.

The findings of our study on the effectiveness of the evaluated bioformulations are similar to the results of previous studies which investigated the effects of some bioformulations on the control of different diseases (Jorjani *et al*. 2012; Kakvan *et al*. 2013). For example, Naraghi *et al*. (2012) evaluated the effectiveness of several bioformulations on the control of Verticillium wilt disease on different crops including cotton, greenhouse cucumber, potato, and tomato and found that most bioformulations were capable of controlling the disease significantly. In another previous study, Jorjani *et al*. (2012) developed some new bioformulations using both inorganic and organic carriers and bacterial antagonists and successfully applied them in the biocontrol of sugar beet seedling damping-off disease. In addition to the above-mentioned studies, in recent research, Kakvan *et al*. (2013) reported the promising effects of several bioformulations developed based on fungal antagonists in combating sugar beet mortality disease.

In our study, bioformulations containing the wild type (phenazine producing) strain of *P. aureofaciens*, performed more effectively than those of mutant strain with no phenazine regulation gene (*phzR*$^-$). This finding proves the important role of phenazine antibiotics in the antagonistic activity of *P. aureofaciens* which has also been demonstrated in previous studies (Pierson III *et al*. 1994; Wood *et al*. 1997). It is worth noting, that mutant strain of *P. aureofaciens* also showed some antagonistic activities both in the laboratory and greenhouse conditions, which can be related to the production of other antibiotics (Dowling and O'Gara 1994).

Accordingly, numerous studies have been performed to find effective carriers for the development of bioformulations for biocontrol bacteria (Amer and Utkhede 2000; Bharathi *et al*. 2004; Chung *et al*. 2005; Mansoori *et al*. 2013; Sallam *et al*. 2013). For this reason, we chose several carriers such as talc powder, rice bran, soybean meal, and cottonseed meal for developing bioformulations of *P. aureofaciens* PhzR$^+$ and PhzR$^-$ in the present study. Data obtained from the greenhouse study showed that the effectiveness of the organic carriers was more than that of the inorganic one. This could be due to the organic nature and carbon sources of such carriers that have also been reported previously (Jorjani *et al*. 2012). For example, soybean meal is known as a rich nitrogen and carbon source. Not only does it have a high concentration of protein (44 to 49%), the protein is highly digestible (NRC 2012). Hence, its carbon and nitrogen sources can be easily available to both beneficial bacteria and host plants. On the other hand, rice bran has a high nutritional value with 12–15% protein content. The protein efficiency ratio (PER) of rice bran is higher than that of soybean meal. Additionally, protein digestibility of rice bran is greater than 90% and can be easily available in the rhizosphere (Wang *et al*. 1999).

The findings of this study clearly indicate that bioformulations developed and used in this research project performed very well and were even more effective than the commonly used fungicide. The overall results of this study are promising and can be used in the biological control of cotton seedling damping off as a component of Integrated Pest Management (IPM) programs for increasing cotton yield, protecting the agricultural environment, and preserving natural resources.

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